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# **Optogenetic manipulation of neural activity with high spatial resolution in freely moving *Caenorhabditis elegans***

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## **Abstract**

**We present an optogenetic illumination system that is capable of real-time light delivery with high spatial resolution to specified cellular targets in freely moving *C. elegans*. In our system, a tracking microscope and high-speed video camera records the posture and motion of an unrestrained worm expressing Channelrhodopsin-2 or Halorhodopsin/NpHR in specific cell types. Custom image processing software analyzes the position of a worm within each video frame, and then rapidly estimates the locations of targeted cells. The software then instructs a digital micromirror device to illuminate targeted cells with laser light of the appropriate wavelengths to stimulate or inhibit activity. Since each cell in an unrestrained worm is a rapidly moving target, our imaging and analysis system operates at high speed (~50 frames per second) to provide high spatial resolution (~30  $\mu\text{m}$ ). To demonstrate the accuracy, flexibility, and utility of our system, we present optogenetic analyses of the worm motor circuit, egg-laying circuit, and mechanosensory circuits that were not possible with previous methods.**

## Introduction

Systems neuroscience aims to understand how neural dynamics create behavior. The recent development of optogenetic techniques based on light-sensitive proteins – e.g., Channelrhodopsin-2 (ChR2) and Halorhodopsin (Halo/NpHR) and a light-gated glutamate channel (LiGluR) – has accelerated progress toward this goal by making it possible to stimulate or inhibit neurons by illuminating them with light <sup>1-7</sup>. The nematode *C. elegans* has been particularly amenable to the development of new optogenetic methods owing to its optical transparency, compact nervous system, relatively simple behaviors, and ease of genetic manipulation <sup>8-11</sup>.

To date, most optogenetic experiments in *C. elegans* have been performed by illuminating the entire animal, thereby triggering changes in membrane potential within all cells expressing light-sensitive channels under the control of cell-specific promoters. However, whole animal illumination spatial selectivity is limited by the availability of cell-specific promoters, and subjects all cells expressing an optogenetic protein to the same temporal pattern of modulation. Guo et al. (2009) recently showed how a digital micromirror device (DMD) can be used to achieve high spatial selectivity in light delivery using immobilized *C. elegans* <sup>10</sup>. Similarly, Wyart et al. (2009) recently used a DMD to locally stimulate LiGluR in immobilized zebrafish spinal locomotor circuits <sup>12</sup>. Each pixel of a DMD can be independently controlled to deliver light to a corresponding pixel of a microscope's field of view, thereby allowing the user to target specific cells within a microscope's field of view. In many cases, however, the normal operation of neural circuits can only be found in freely moving animals exhibiting unrestrained behavior. Moreover, determining the roles of specific neurons in generating different behaviors requires quantification of these behaviors while perturbing neural activity. Thus, optogenetic analysis of neural circuit function would be greatly enhanced by an illumination system that allows arbitrary spatiotemporal perturbations of cellular activity throughout the body of a freely moving animal.

Here, we describe the development and application of a new optogenetic illumination system that allows us to control locomotion and behavior in real time (CoLBeRT) in *C. elegans*. In the CoLBeRT system, a video camera follows the movements of an individual worm under dark field illumination, and a motorized translation stage automatically keeps the animal centered in the camera's field of view. Streamlined machine-vision algorithms rapidly estimate the coordinates of targeted cells within the worm body, and an illumination pattern is rapidly calculated. Finally, the illumination pattern is projected onto the worm with either 473 nm laser light (to stimulate ChR2) or 532 nm laser light (to stimulate Halo/NpHR) using a DMD, and the cycle repeats itself for the next frame. Because a freely moving worm is in constant motion, every image of the worm is already out of date by the time it arrives in the computer. The faster an image can be captured, processed and translated into DMD commands, the more accurately an individual neuron can be targeted. The CoLBeRT system currently performs all of these functions in ~20 ms, and provides ~30 micrometer spatial resolution in optogenetic control when tracking a swimming *C. elegans*.

The high spatial resolution afforded by the CoLBeRT system now allows optogenetic perturbations of cellular dynamics in cases that genetic methods do not provide adequate cellular specificity. In the worm motor circuit, for example, promoters have not yet been identified that drive expression in only one or a few neurons of the ventral nerve cord. We note that there are genetic strategies for sparse gene expression in *C. elegans* (e.g., mosaic gene expression), but it is often preferable to be able to separately perturb different cells within the same transgenic animal<sup>13</sup>. Optogenetics has been applied to the mechanosensory circuit in *C. elegans*, but again because promoters that individually label the six mechanosensory neurons are unavailable, only by illuminating all mechanosensory neurons at once<sup>1</sup>. Laser killing allows one to study the contribution of single touch receptor cells to overall behavior by removing cells and quantifying the contribution of the remaining circuit, but it is often preferable to work with intact circuits

14-16 .

Here, we carry out a series of studies on the motor circuit and mechanosensory circuit of freely moving animals that illustrate the performance of the CoLBeRT system, a powerful new tool for *C. elegans* neurophysiology.

## Results

### *Laser illumination system*

The hardware layout of our system is shown in Figure 1A. To either stimulate neurons using channelrhodopsin-2 (ChR2) or inhibit neurons using Halorhodopsin (Halo/NpHR), we employ a 473 nm or 532 nm diode-pumped solid state (DPSS) laser, respectively. Either laser is incident onto a DMD with 1024x768 elements, such that only when an individual element is turned to the ‘on’ position is blue or green light reflected into the microscope and onto the specimen. The microscope condenser is configured to provide dark-field illumination of the specimen through a red filter (to avoid exciting ChR2 or Halo/NpHR). Filter cubes reflect the shorter wavelengths for optogenetic illumination from the DMD onto the sample, while passing longer wavelengths for dark-field illumination from the specimen to an imaging camera. The microscope is also outfitted with an automated XY translation stage that, driven by software, automatically keeps the worm within the center of the field of view at all times.

### *Software*

To make real-time image analysis of worm position and posture as fast as possible, we developed the *MindControl* software package using the open-source OpenCV computer vision library<sup>17</sup>. To make *MindControl* easy to use, we designed an intuitive graphical user interface (GUI).

*MindControl* enables the user to design sets of illumination patterns targeting specific regions of freely moving animals, as well as trigger these illumination patterns either manually or automatically according to predefined sequences. The software, installation instructions, and user guide are freely available for download at [colbert.physics.harvard.edu](http://colbert.physics.harvard.edu).

The basic sequence of image analysis operations carried out by *MindControl* is shown in Figure 1B. Each image from the camera is captured by the computer, filtered to reduce noise, and thresholded. Next, the boundary of the worm is calculated, and head and tail are identified by calculating local maxima of boundary curvature (the head is blunt and the tail is sharp). The worm's centerline is calculated and the worm's body is divided into 100 evenly spaced segments. These segments define a worm coordinate system that is invariant to instantaneous worm posture or orientation, within which the user may define the position of targets. The software maps the position of targets onto the coordinates of the real image, and finally sends the appropriate pattern to the DMD for illumination.

For our current system, we have characterized the time required for each step between image acquisition and DMD illumination:

Image exposure	2 ms
Image data transfer to computer	3 ms
Image analysis and transformation	10 ms
<u>Data transfer to the DMD</u>	<u>5 ms</u>
<b>Total latency</b>	<b>20 ms</b>

Given the size and typical speed of a swimming worm tracked at 10x magnification, our system working at ~50 fps is capable of optogenetic illumination with ~30 micrometer spatial resolution. We note that the CoLBeRT system is not far from the limit to spatial resolution (~5 micrometers at 10x magnification) which is limited by the pixel density of the DMD. Below, we describe experiments that demonstrate the performance of the CoLBeRT system as a new tool in *C. elegans* neurophysiology.

*Illumination is restricted to targeted area*

First, we examined a transgenic worm that expresses Halor/NpHR under the *myo-3* promoter in all body wall muscles. Whole animal illumination of transgenic *Pmyo-3::Halo/NpHR* worms abolishes locomotion by causing all muscles to relax<sup>6</sup>. We placed individual swimming *Pmyo-3::Halo/NpHR::CFP* transgenic worms in the CoLBeRT system, and used green light (532 nm, 10 mW/mm<sup>2</sup>) to alternately illuminate the entire region outside and inside the worm boundary. Illuminating the entire region outside the worm boundary had no effect on locomotion as bending waves continued to propagate from head to tail at normal speed (Fig 2a and Supplementary Movie 1). Illuminating the entire region inside the worm boundary, however, arrested locomotion as the body of the swimming worm relaxed and the speed of bending waves dropped to zero (Fig 2a and Supplementary Movie 1).

### *Quantifying spatial resolution*

To quantify the spatial resolution of the CoLBeRT system, we measured its targeting accuracy in evoking egg-laying events in transgenic worms expressing ChR2 in the HSN motor neurons. Optogenetic stimulation of the bilaterally symmetric HSN neurons, which innervate the vulval musculature, robustly evokes egg-laying behavior in gravid *C. elegans* (Niels Ringstad, personal communication). We used transgenic worms expressing ChR2 under the *egl-6* promoter, which drives expression in the two bilaterally symmetric HSN neurons (HSNL and HSNR) as well as glia-like cells in the worm head<sup>18</sup>. The two HSN neurons lie on top of one another when the worm is viewed from above as it undulates in its dorsal/ventral plane, so our system necessarily targets both neurons.

For these experiments, we projected a thin stripe of blue light (473 nm, 5 mW/mm<sup>2</sup>) against the body of freely moving *Pegl-6::ChR2* transgenic worms. The long axis of the stripe was orthogonal to the worm centerline and spanned the full worm diameter. The width of the stripe corresponded to 2% of the anterior-posterior length of the centerline of each worm (i.e., ~20 micrometers of the ~1 mm long young adult worm). We slowly moved the illumination stripe along the centerline of freely moving worms while recording egg-laying events. Of 14 animals



that were studied, we observed 13 egg-laying events, 8 where the stripe started at the head and moved backward, and 5 where the stripe started at the tail and moved forward. We found that the frequency of egg laying events was sharply peaked when the center of the stripe coincided with the centerline coordinate of HSN, or 49.6% of the total distance from the anterior tip to the posterior tip of the worm body with 3.2% standard deviation (Fig. 2B; Supplementary Movie 2). The width of this distribution suggests that the CoLBeRT system provides at least ~30 micrometers of spatial resolution in an unrestrained worm.

### *Analysis of the worm motor circuit - optogenetic manipulation of muscle cells*

The neuromuscular circuit that generates worm locomotion remains poorly understood in comparison to better studied undulatory animals like the leech and lamprey<sup>19-21</sup>. In *C. elegans*, forward movement is generated by the activity of 58 motor neurons in the ventral nerve cord (VNC) that coordinate the activity of 95 body wall muscle cells along its dorsal and ventral sides<sup>22</sup>. Because the normal operation of this neuromuscular circuit is only likely to occur during normal movement, technology like the CoLBeRT system has been required to dissect cellular activity in unrestrained animals.

We used the CoLBeRT system to suppress muscle activity in one segment of the motor circuit of *myo-3::Halo/NpHR::CFP* transgenic animals. To graphically represent this perturbation of undulatory dynamics, Fig 3a is a kymograph that quantifies the curvature of the body centerline in non-dimensional units (i.e., the curvature calculated at each point along the centerline,  $\kappa$ , multiplied by worm length,  $L$ ) as a function of time and fractional distance along the centerline,  $s$ , from head ( $s = 0$ ) to tail ( $s = 1$ ) (Supplementary Movie 3). Interestingly, hyperpolarizing muscle cells in one segment had no effect on undulatory dynamics anterior to the segment, but lowered the amplitude of the bending wave posterior to the illuminated segment (Fig. 3b). This observation suggest that the bending of posterior body segments is directly coupled to the bending of anterior body segments. One possibility is that muscle activity in posterior segments is directly promoted by muscle activity in anterior segments, perhaps by gap junction coupling

between muscle cells <sup>23</sup>. Another possibility is that the motor circuit contains a proprioceptive mechanism that makes the activity of posterior segments directly sensitive to the bending of anterior segments. If so, neurons and/or muscle cell in *C. elegans* must be sensitive to their own stretch or displacement by mechanisms that remain to be determined.

#### *Analysis of the worm motor circuit - optogenetic manipulation of cholinergic motor neurons*

Forward locomotion in *C. elegans* is driven by four neuronal types whose cell bodies are distributed along VNC <sup>14</sup>. Ventral muscles are innervated by the cholinergic VB motoneurons and GABAergic VD motoneurons. Dorsal muscles are innervated by dorsal projections of the cholinergic DB motoneurons and GABAergic DD motoneurons <sup>24</sup>. Cholinergic motoneurons and GABAergic motoneurons drive muscle contraction and relaxation, respectively. The pattern of synaptic connectivity between these neuronal types allows for contralateral inhibition, such that DB motor neurons can simultaneously excite a dorsal muscle cell while exciting VD motor neurons that inhibit the opposing ventral muscle cell, and vice-versa <sup>24,25</sup>. This repeating motif of synaptic connectivity between these four neuronal types runs along the worm body. However, how this motoneuron network generates, propagates, and organizes the rhythmic undulatory wave remains poorly understood.

VB and DB represent the cholinergic neurons that are dedicated to forward movement in *C. elegans* <sup>26</sup>. We used the CoLBeRT system to analyze the contributions of VB and DB to undulatory dynamics using transgenic worms expressing Halo/NpHR under the control of the *unc-17* promoter, which drives expression in all cholinergic neurons <sup>27</sup>. When we illuminated only a short segment of the VNC of *Punc-17::Halo/NpHR::CFP* transgenic worms, we found that we could suppress propagation of the undulatory wave to the entire region posterior to the illuminated segment without affecting the generation or propagation of the undulatory wave anterior to the illuminated segment (Fig. 4a, b; Supplemental Video 4). This observation suggests that the activity of posterior VB and DB neurons is coupled to the activity of anterior

VB and DB neurons during forward locomotion, consistent with a wave of neuronal excitation that propagates from head to tail during forward movement.

The CoLBeRT system also allows us to specifically illuminate either the dorsal nerve cord or the ventral nerve cord (Supplemental Video 5). As expected, illuminating the entire dorsal nerve cord had no effect on undulatory dynamics whereas illuminating the entire ventral nerve cord, which contains all the cell bodies of the VB and DB neurons, induced paralysis. Surprisingly, paralysis occurred without allowing relaxation of the worm body. Instead, as long as the entire cholinergic network within the VNC was deactivated, the worm remained locked in its posture immediately before illumination (Fig 4c, d). When the muscle cells of a swimming are directly hyperpolarized, on the other hand, the body straightens (Supplementary Video 1). We note that making this comparison required using a closed-loop optogenetic illumination that was sufficiently fast to follow the movements of a freely swimming worm. This observation suggests that muscle cells can remain in contracted or relaxed states without requiring continuous cholinergic input, which is consistent with a theoretical model of worm undulatory dynamics that involves positive feedback between muscle stretch and muscle activity (Q. Wen, M. Wyart, and D. Chklovskii, personal communication).

#### *Analysis of the worm mechanosensory circuit – optogenetic manipulation of single touch receptor types*

Next, we applied the CoLBeRT system to the touch receptor system in *C. elegans*. Six microtubule cells are specialized for sensing gentle touch in *C. elegans*: the left and right anterior lateral microtubule cells (ALML and ALMR); the left and right posterior lateral microtubule cells (PLML and PLMR); the anterior ventral microtubule cell (AVM); and the posterior ventral microtubule cell (PVM) <sup>14</sup>. Gently touching the worm near its anterior stimulates reversal movement dependent upon ALML, ALMR, and AVM. Gently touching the worm near its posterior stimulates forward movement dependent upon PLML and PLMR. Interestingly, PVM does not mediate touch response, and its role in the worm nervous system remains unclear. A

small circuit of interneurons and command interneurons integrate information from ALM, AVM, and PLM to transduce touch receptor activity into forward and backward movement.

Early experiments laying the groundwork for optogenetics in *C. elegans* were directed at the touch receptor circuit<sup>1</sup>. Channelrhodopsin can be expressed in all six touch receptor cells using the *mec-4* promoter. Illuminating transgenic animals to blue light evoked reversal responses, presumably by simultaneously activating ALM, AVM, and PLM<sup>1</sup>. The fact that the worm moved backward rather than forward might suggest that ALM and AVM can outweigh PLM in driving the downstream circuit.

The spatial resolution afforded by the CoLBeRT system allowed us to individually activate the ALM, AVM, and PLM cell types. Because the worm lies on its side, the left and right lateral cells (ALML and ALMR; PLML and PLMR) lie on top of one another when viewed from above. Illuminating the anterior end containing both the AVM and ALM neurons triggered reverse movement (Fig. 5a; Supplementary Movie 6). Illuminating the posterior end containing the PLM neurons triggered forward movement (Fig. 5b; Supplementary Movie 8).

The CoLBeRT system also enabled us to trigger reversals by targeting just AVM or ALM with an illumination box (20 micrometers in dorsal ventral width; 30 micrometers in anterior posterior length for a typical young adult worm) that was centered on each cell body (Fig. 5c, d; Supplementary Movies 9, 10). These illumination boxes enabled us to avoid hitting the axon of the other neuron. These observations are consistent with the discovery by Chalfie et al. (1985) that single touch receptor types are sufficient to drive behavioral responses<sup>14</sup>.

Nagel et al. (2005) showed that the mechanosensory circuit habituates to repetitive optogenetic stimulation<sup>1</sup>. We used the CoLBeRT system to quantify the rate of AVM and ALM habituation over 40 min by repeatedly stimulating either AVM or ALM every 60 seconds. We observed comparable rates of habituation for both ALM and AVM (Fig. 6a, b). Kitamura et al. (2001) studied loci for habituation in the mechanosensory circuit by laser killing touch receptor cells

and/or downstream neurons and quantifying rates of habituation to gentle touch<sup>16</sup>. If habituation partly occurs at interneurons that are downstream of both ALM and AVM (e.g., the AVD interneuron), then one might expect cross-habituation of the AVM response to repeated ALM stimulation, and vice-versa. To test this possibility, we subjected an animal to interleaved AVM and ALM stimulation every 30 s, such that each neuron type was stimulated every 60 s. We found that the rates of habituation to both AVM and ALM stimulation were indeed more rapid with interleaved stimulation than with individual stimulation. This effect was particularly dramatic in the case of AVM stimulation (Fig. 6c).

## Discussion

The anatomical simplicity, genetic tractability, optical transparency, and small size of *C. elegans* makes it an ideal animal for the development and application of optogenetic methods. The CoLBeRT system greatly enhances the flexibility and power of optogenetic approaches in the neurophysiological analysis of worm behavior by introducing high spatial and temporal resolution in the study of freely moving animals.

The CoLBeRT system may be adapted to the optogenetic analysis of other genetically tractable, transparent animals like the *Drosophila* or zebrafish larva<sup>28-30</sup>. A simplified version of the CoLBeRT system (without the closed loop between image analysis of animal posture and delivery of spatial illumination) may also be used to facilitate optogenetic illumination in other settings, e.g., studies of mammalian brain slices or exposed brain surfaces. Variants of the CoLBeRT system utilizing its capacity for rapid closed-loop feedback may be used to trigger optogenetic stimulation based on simultaneous recordings of neural activity, not animal posture.

In summary, the CoLBeRT system represents a powerful new tool for optogenetic analysis of neural circuits, providing a flexible and easy-to-use platform to design and project arbitrary spatiotemporal patterns of illumination with closed-loop sensitivity to the real-time behavior of the organism.

## Methods

### *Strains*

Transgenic worms were cultivated in the dark at 20 °C on nematode growth medium (NGM) plates with OP50 bacteria with all-*trans* retinal. OP50-retinal plates were made by seeding 6 cm NGM plates with 250 ul of a suspension of OP50 bacteria in LB, to which 1 ul of 100 mM retinal in ethanol was added immediately prior to seeding. Plates were stored in the dark and handled in the dark or under red light.

The strain FQ10 (*Pegl-6::Halo::GFP*) was a gift of Niels Ringstad (NYU School of Medicine, New York, NY). The strains ZX444 (*lin-15(n765ts); zxE29[pmyo-3::NpHR::ECFP;lin-15+]*) and ZX422 (*lin-15(n765ts);zxE33[punc-17::NpHR::ECFP;lin-15+]*) were gifts of Alexander Gottschalk (Frankfurt Molecular Life Sciences Institute, Frankfurt, Germany). The strain *Pmyo-3::Halo::CFP* used in our experiments was generated by integrating the transgene in ZX444 by cobalt-60 irradiation and outcrossing the resulting strain 3X to the wild-type N2 strain. The strain *Punc-17::Halo::CFP* used in our experiments was generated by Mei Zhen (Samuel Lunenfeld Institute, Toronto, Canada) by irradiating ZX422 using UV radiation and outcrossing 2X to the wild-type N2 strain. The *Pmec-4::ChR2* strain (QW309) was generated by injection of *Pmec-4::ChR2::YFP* plasmid at 100 ng/μl into *lin-15(n765ts)* animals along with the *lin-15* rescuing plasmid (pL15 EK) at 50 ng/μl; the extrachromosomal array was integrated using gamma irradiation and outcrossed 4 times to wild-type N2.

### *Microscopy*

The setup is built around a Nikon Eclipse TE2000-U inverted microscope. Dark field imaging was performed using annular illumination of the specimen through a Ph3 phase ring. A red-light

transmitting filter (Hoya) was mounted to the microscope illumination optical pathway in order to minimize inadvertent activation of ChR2 and/or Halo/NpHR due to dark field illumination.

Worms were imaged using a 10X/ NA 0.45 Plan Apo objective. We used a custom optical system composed of two camera lenses (Nikon) to reduce the size of the image on the camera by a factor of 3.5. This allowed us to capture almost all of the 2.5mm diameter field of view on the camera sensor. A PhotonFocus MV2-D1280-640CL camera and BitFlow Karbon PCI Express x8 10-tap Full Camera Link frame grabber were used to capture images.

The microscope stage was controlled by a Ludl BioPrecision2 XY motorized stage and MAC 6000 stage-controller. During data acquisition, computer software kept the worm centered in the field of view via an automated feedback loop.

### ***Optics and illumination***

To stimulate ChR2 we used a diode-pumped solid state (DPSS) laser (LP473-100, 473 nm wavelength, 100mW maximum power, LaserShowParts, Shanghai, China). Similarly, to stimulate Halo/NpHR we used a DPSS laser (LP532-200, 532 nm wavelength, 200mW maximum power, LaserShowParts). The beams from the two lasers are aligned to a common path by a dichroic beamsplitter, but for each experiment only one of the two lasers is used.

The laser beam is expanded using a telescope composed of two plano-convex lenses and incident onto a 1024 x 768 digital micromirror device (Texas Instruments DLP, Digital Light Innovations, Discovery 4000 BD VIS 0.55" XGA) attached to a mirror mount. Using a series of mirrors, the laser is aligned such that the reflected beam for the "ON" state of the DMD is centered on the optical axis of the illumination pathway.

The plane of the DMD was imaged onto the sample via the epifluorescence illumination pathway of microscope using an optical system composed of two achromatic doublet lenses. A custom

dichroic filter (Semrock, Rochester, NY) was used to reflect 473 nm or 532 nm onto the sample while passing the longer wavelengths of dark field illumination ( $>600$  nm). An emission filter was used to prevent stray laser reflections from reaching the camera. The dichroic and emission filters were mounted in a custom filter cube in the microscope filter turret.

## ***Software***

The microscope and all its components were controlled with custom *MindControl* software running Windows XP on an Acer Veriton M670G quad-core computer with four Intel Core2 2.83 GHz processors and 3GB of RAM. *MindControl* enables the user to define arbitrary illumination patterns for optogenetic stimulation, and to deliver illumination patterns either manually or automatically. To operate rapidly, *MindControl* was written in the C Programming Language utilizing the open source OpenCV computer vision library along with Intel's Integrated Performance Primitives for maximal speed. To further increase speed, multiple threads were used to separately handle image processing and the user interface. Every 20 ms, *MindControl* acquires an image from the camera, computes the location of the worm, generates an illumination pattern, and sends that pattern to the DMD. For each video frame, the boundary and centerline of the worm and the status of the stimulus is recorded in a human- and computer-readable YAML file. Every frame is also recorded in two video streams, one containing annotations about optogenetic stimulation, and the other containing just images of the freely moving worm. A graphical user interface allows the user to adjust the parameters of optogenetic stimulation in real time during each experiment. After each experiment, quantitative analysis of video records was performed using custom scripts written in MATLAB. All software and documentation is freely available for modification and redistribution under the GNU Public License and can be downloaded from [colbert.physics.harvard.edu](mailto:colbert.physics.harvard.edu).

## ***Behavioral experiments***



For each experiment, we prepared worms in a darkroom under red light conditions. To study the motor circuit, young adult worms were washed in NGM solution and transferred to a slide prep consisting of a 30% dextran in NGM solution sandwiched between two microscope slides spaced 0.127 mm apart so that the worm was approximately confined to two dimensions but otherwise able to move freely. The slide was then placed in the microscope for tracking and optogenetic analysis.

To analyze egg-laying, gravid young adult worms were selected, washed in NGM, and transferred to the slide prep. Each worm was then subject to sequential pulses of 4 second blue light illumination. Each pulse illuminated a stripe orthogonal to the worm centerline, spanning the worm diameter with width corresponding to 2% of total body length. The stripe progressed along the worm centerline from head to tail or from tail to head until the first egg was laid. After an egg was laid, the trial ended and the worm was discarded. No eggs were laid by only one out of fourteen worms that were studied.

To analyze the mechanosensory response, young adult worms were prescreened on a Nikon SMZ 1500 fluorescence stereomicroscope by illuminating the anterior of the worm with blue light from a 50 W mercury lamp filtered through the GFP excitation filter. Only worms that responded with a reversal were chosen for the experiment. Worms were then transferred to a clean NGM plate and allowed to crawl for ~30 s to free themselves of bacteria. Worms were then transferred onto a slide with a thin layer of NGM agar, covered with a drop of mineral oil, then covered with a slide. Specific regions of each worm were targeted with blue light and illuminated for 1.5 s. Anterior touch responses were scored by quantifying the bending wave speed 2 s before stimulus onset and 3 s after stimulus onset. A successful response was classified as a reduction in wave speed by  $>0.03$  body-length per second.

#### *Quantifying locomotory gait*

The locomotory behavior of individual worms was analyzed by quantifying time-varying worm posture in each video sequence. A least-squares cubic smoothing spline fit to the body centerline was calculated, and curvature was calculated at each point along the centerline as the derivative of the unit vector tangent to the centerline with respect to the distance along the centerline. To graphically display locomotory gait, we utilize kymographs of curvature as a function of distance along the centerline and time. The speed of the bending wave along the centerline within the reference frame of the worm body was calculated by measuring the displacement of curvature profiles along the centerline ( $\Delta x$ ) at successive points in time ( $\Delta t$ ) according to  $v = \Delta x / \Delta t$ .

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## FIGURE LEGENDS

### Figure 1 | Optogenetic control with high spatial resolution in freely moving *C. elegans*

(a) Schematic of the hardware layout of the CoLBeRT system. The worm swims or crawls on a motorized XY tracking stage under darkfield red light illumination. A high-speed camera monitors the worm's location. Custom computer software instructs a DMD to reflect 473nm or 532nm laser light onto targeted cells.

(b) Sequence of image-processing steps carried out by the *MindControl* software package. A 1024x768 pixel grayscale image is acquired at ~50 frames per second. The image is thresholded and the boundary is found. The head and tail are identified based on local maxima of boundary curvature (red arrows). A centerline is extracted at the midpoint of a line segment that connects a point on the dorsal boundary with the nearest point on the ventral boundary (blue bar). The worm's body is partitioned into 100 equidistant segments by finding a set of vectors (green arrows) that originate at each point along the centerline and end on the boundary, and selecting that vector (orange arrow) that lies perpendicular to the boundary. An illumination pattern is sent to the DMD by mapping targets defined in the worm coordinate system to the video image (green bar).

### Figure 2 | Spatial resolution of the CoLBeRT system

(a) Body wall muscles in *C. elegans* line the dorsal and ventral sides of the animal as shown in the schematic. The bending wave speed of a swimming worm expressing Halo/NpHR in all body wall muscles is shown as the worm is subjected to green light illumination outside the worm boundary or inside the worm boundary (n=5 worms, representative trace).

(b) The HSN neuronal cell bodies are near the middle of the animal as shown in the schematic. The histogram shows the position at which a narrow stripe of blue light that was slowly scanned along the centerline of a transgenic *Pegl-6::ChR2* worm stimulated an egg-laying event (n=13

worms).

### Figure 3 | Optogenetic inactivation of muscle cells

(a) A kymograph of time-varying body curvature along the centerline of a *Pmyo3::Halo/NpHR* transgenic worm. Between  $t = 0$ s and  $t = 4$ s, the worm is stimulated with green light (10 mW/mm<sup>2</sup>) in a region spanning the full worm diameter and between 0.38 and 0.6 of the fractional distance along the worm's centerline ( $n = 5$  worms, representative trace).

(b) For the kymograph shown in (a), time-varying curvature is shown at specific points along the worm centerline anterior (upper panel) and posterior (lower panel) to the illuminated region.

### Figure 4 | Inhibition of Motor Neurons

(a) The schematic shows the positions of the cholinergic DB and VB motor neurons that contribute to forward movement. A kymograph of time-varying body curvature along the centerline of a *Punc-17::Halo/NpHR* transgenic worm that is subjected to a stripe of green light along its ventral nerve cord between  $t = 0$ s and 1.6s. In the dorsal-ventral direction, the stripe width was equal to 50% of the worm diameter and centered on the ventral boundary. In the anterior-posterior direction, the stripe length was between 0.14 and 0.28 of the fractional distance along the worm body ( $n = 5$  worms, representative trace).

(b) For the kymograph shown in (a), time-varying curvature is shown at specific points along the worm centerline anterior (upper panel) and posterior (lower panel) to the illuminated region.

(c) Video montage showing a worm subjected to a long stripe of green light spanning the ventral nerve chord between  $t = 0$ s and 1.8s.

(d) The bending wave speed of a swimming worm subjected to a long stripe of green light lasting 1.8s spanning the ventral nerve cord (upper panel) and dorsal nerve cord (lower panel) ( $n = 10$  worms, representative trace).



## Figure 5 | Optogenetic analysis of mechanosensory neurons

The schematic shows the positions of the anterior and posterior touch receptor cell types. Kymographs (left panels) show the time-varying curvature of the worm centerline of *Pmec-4::ChR2* transgenic worms subjected to rectangles of blue light (5 mW/mm<sup>2</sup>) that target different groups of touch receptor neurons. Plots of bending wave speed (right panels) indicate stimulus-evoked changes in direction or speed.

**(a)** The AVM and ALM neurons are subjected to 1.5 s of stimulation with a rectangular illumination with width spanning the full diameter of the worm and length between 0 and 0.46 of the fractional distance along the worm centerline ( $n = 5$  worms, representative trace).

**(b)** The PVM and PLM neurons are subjected to 2.5 s of stimulation with a rectangular illumination with width spanning the full diameter of the worm and length between 0.62 and 0.99 of the fractional distance along the worm centerline ( $n = 5$  worms, representative trace).

**(c)** The ALM cell body is specifically stimulated by illuminating a small rectangle. The width of the rectangle was between 0.3 of the fractional distance between the centerline and dorsal boundary and 0.9 of the fractional distance between the centerline and ventral boundary. The length of the rectangle was between 0.38 and 0.46 of the fractional distance along the length of the worm ( $n=14$  worms, representative trace).

**(d)** The AVM cell body is specifically stimulated by illuminating a small rectangle. The width of the rectangle was between 0.3 of the fractional distance between the centerline and dorsal boundary and 0.9 of the fractional distance between the centerline and ventral boundary. The length of the rectangle was between 0.30 and 0.38 of the fractional distance along the length of the worm ( $n=14$  worms, representative trace).

## Figure 6 | Habituation of Individual Mechanosensory Neurons

Individual ALM and AVM mechanosensory neurons are repeatedly stimulated with blue light for 1.5s every 60s for ~40 min, either alone **(a,b)** or interleaved within each experiment **(c)** as illustrated above each plot. The fractional response to stimulation of each neuronal type is

measured over the course of 40 min and fit to an exponential of the form  $a + b \exp[-t/\tau]$  using the maximum likelihood estimator. The time constant for habituation,  $\tau$ , is extracted from each fit.

**(a)** Fractional response of ALM when stimulated alone ( $n = 7$  worms). **(b)** Fractional response of AVM neuron when stimulated alone ( $n = 7$  worms). **(c)** Fractional response of ALM (left panel) and AVM (right panel) during interleaved stimulation of both neurons ( $n=7$  worms).

## SUPPLEMENTAL MOVIE CAPTIONS

### Supplementary Movie S1

A *Pmyo-3::Halo::CFP* worm expressing Halo in muscle is induced to relax only when the CoLBeRT system illuminates within the worm's body. During frames 6707-6771, the entire region outside the worm's boundary is illuminated with green light and the worm continues locomotion. During frames 6847-6917, only the region inside the boundary of the worm is illuminated and the worm relaxes. During frames 7052-7117 only the region outside the worm's boundary is illuminated and the worm continues locomotion. The frame number is indicated in the bottom right hand corner. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

### Supplementary Movie S2

An *Pegl-6::Chr2::GFP* worm is induced to lay eggs when a stripe of blue light reaches HSN. A narrow stripe of light, 0.02 of the fractional length along the worm centerline and twice the width of the worm, progresses from the worm's head towards its tail. The stripe takes steps of 0.02 fractional worm lengths and illuminates for 4s at each step. At frame 8828, the illumination band reaches HSN and the worm lays eggs. The frame number is indicated in the bottom right hand corner. Bright white shading indicates the illumination target and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

### Supplementary Movie S3

The bending waves of a *Pmyo-3::Halo::CFP* transgenic are dampened and the anterior relaxes when a portion of the worm is illuminated with blue light. The illumination is turned on 4.8s into

the movie. The worm recovers after the illumination is turned off. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

#### **Supplementary Movie S4**

The bending waves of an *Punc-17::Halo::CFP* are abolished when a small ventral region near the worm's head is illuminated. During frames 9075 to 9141, the worm is illuminated and no bending waves are propagated from the head to the tail. On the contrary, the worm posterior to the region of illumination is paralyzed and the curvature is frozen. Only after the stimulation ends, are bending waves again able to propagate from the anterior to posterior of the worm. The frame number is indicated in the bottom right hand corner. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

#### **Supplementary Movie S5**

An *Punc-17::Halo::CFP* transgenic worm is paralyzed only when the ventral nerve cord is illuminated (frames 37909-37971), but not when the dorsal nerve cord is illuminated (frames 38233-38295). Note how during paralysis the worm does not relax to a neutral position, but rather it retains its curvature even while the motoneurons are inhibited. In this video, a small circle identifies the worm's tail and a large circle identifies the worm's head. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area.

#### **Supplementary Movie S6**

The anterior of a *Pmec-4::ChR2::GFP* worm is illuminated for 1.5s and the worm undergoes a reversal. During frames 7645-7709, the anterior 46% of the worm is illuminated, which includes

the neurons AVM and ALM and their associated processes. The frame number is indicated in the bottom right hand corner. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

### **Supplementary Movie S7**

The posterior of a *Pmec-4::ChR2::GFP* worm is illuminated for 1.5s and the worm accelerates from a resting position. During frames 13655-13733, the posterior 38% of the worm is illuminated, which includes the neurons PVM and PLM and their associated processes. The frame number is indicated in the bottom right hand corner. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

### **Supplementary Movie S8**

The cell bodies of ALM are illuminated for 1.5s in a *Pmec-4::ChR2::GFP* worm during frames 2013-2079 and the worm undergoes a reversal. The frame number is indicated in the bottom right hand corner. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.

### **Supplementary Movie S9**

The cell body of the single neuron AVM is illuminated for 1.5s in a *Pmec-4::ChR2::GFP* worm during frames 1925-1994 and the worm undergoes a reversal. The frame number is indicated in the bottom right hand corner. Light white shading indicates the area where the system is targeting. Bright white shading and the words “DLP ON” indicate that the system is illuminating

the targeted area. A small circle identifies the worm's head and a large circle identifies the worm's tail.